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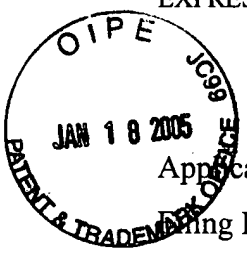


**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Clinton et al.
Filing Date: 16 February 2000
Serial No.: 09/506,079
For: HER-2 BINDING ANTAGONISTS
Art Unit: 1642
Examiner: Anne L. Holleran
Docket: 49321-16
Date: 18 January 2005

Mail Stop Amendment
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

**AFFIDAVIT OF DR. GAIL M. CLINTON UNDER 37 C.F.R. § 1.132**
(IN SUPPORT OF RESPONSE AND AMENDMENT UNDER37 CFR § 1.111)

Sir or Madam:

I, Dr. Gail Clinton, being duly sworn, say:

1. I am an inventor of the subject matter described in the above-identified pending patent application.

2. I am presently employed as an Associate Professor at Oregon Health and Science University in Portland, Oregon (from 1/01/87 to present). I received a Bachelor of Science Degree in 1969 from the University of California, San Diego, and a Ph.D. degree from the University of California, San Diego in 1974. I completed a postdoctoral fellowship at Harvard Medical School in 1981.

3. I am an author or co-author of more than 50 peer-reviewed research articles in the field of oncogene regulation and I am a member of a number of scientific and medical societies, most notably American Association of Cancer Research. I have received a number of prizes and

awards for achievement in research. Most notably, I was the recipient of a postdoctoral fellowship from the American Cancer Society, numerous grants from the National Cancer Institute, and was awarded a Fogarty Senior International Fellowship. I have served on several peer review groups and study sections and have been invited to give numerous presentations on my research at national and international meetings.

4. I have read the above-identified patent application, and the attached Response and Amendment, and it has been explained to me that particular claims have been rejected by the Patent Examiner based on an alleged lack of enablement for binding and biological activity of various Herstatin polymorphisms: variants 1-10 (corresponding to SEQ ID NOS:19-28 and 29-38); and the "most common sequence" (wild-type "wt" sequence) shown in Figure 8 of the present application (corresponding to SEQ ID NOS:14 and 15). I understand that the Examiner is questioning whether, apart from the Herstatin that was previously disclosed and claimed (variant 11), the Herstatin polymorphisms (including the most common sequence) ~~actually~~ bind to HER-2 and inhibit its activity.

5. This data provided in this affidavit was obtained under my supervision and follows the teachings of the present application and ~~the~~ standard methods as described herein. Some of the data has been published subsequent to the present application (*i.e.*, Shamieh L and Clinton GM, *FEBS Lett*, vol 568, pp163-166 (2004) (attached hereto). A comparison of the binding and biological activities among several Herstatin polymorphic forms, two of which (i.e., variant 11 and the "wt" sequence) are described in the present application, ~~and~~ shows that Herstatin allelic variants have comparable HER-2 binding and biological activity in inhibiting HER-2 dimerization on cancer cells.

6. *Materials and Methods.*

***Cells***—HER-2 overexpressing 3T3 cells are known in the art (*e.g.*, Lin and Clinton, Oncogene 6:639-643, 1991) and are the same as those used in the present application.

***Herstatin proteins***—The alleles studied are wild-type ("wt"), "L/N", and "R to C". Additionally, a rare mutant referred to in Shamieh & Clinton as "R to I" was investigated. Wild-type "wt" corresponds to the most common sequence (*i.e.*, to that of Figure 8 of the present application, corresponding to present SEQ ID NOS:14 and 15). The "L/N" variant corresponds to the previously claimed variant 11 and contains two substitutions (double variant) in the intron 8 encoded ECDIIIa domain; namely Pro to Leu, and Asp to Asn at amino acids positions 6 and 73, respectively, relative to the "wt" form. The "R to C" variant contains a substitution of Cys in place of Arg at amino acid position 17 of the intron encoded region. The "R to I" encoded ECDIIIa allelic variant with a Arg –> Ile substitution at amino acid position 31 of the ECDIIIA region.

***Purification of proteins***—To obtain polypeptides corresponding to the intron 8 – encoded region of Herstatin, the various polymorphic intron 8 cDNAs were expressed as poly-His proteins using a pET30 bacterial expression vector and BL-21 cells. (IS THIS SENTENCE NEEDED? IS THERE DATA IN THIS DECLARATION THAT USES THE INTRON 8-ENCODED POLYPEPTIDES?) To obtain full length Herstatin (and variants), the "wt" Herstatin (with an N-terminal poly Histidine tag) in an insect expression vector, was mutagenized to generate the "L/N," "R to C, and R to I " forms.. S2 insect cells were stably transfected with the "wt" and mutant forms, their expression was induced by copper, and each was purified by nickel affinity chromatography.

***Binding assays***—Binding of Herstatin polymorphic proteins to HER-2 overexpressing 3T3 cells was performed by standard methods as taught by the present application. The binding comparison was done by adding 100 or 500 nM protein to HER-2 overexpressing 3T3 cells or to parental 3T3 cells, incubating the cells on ice for 2 hrs, washing the cells twice with PBS, extracting the cell cultures, and measuring the bound Herstatin by Herstatin-specific ELISA (Upstate) as described in Shamieh L and Clinton GM, *FEBS Lett*, vol 568, pp163-166 (2004) (attached hereto). The results (Figure 1) are plotted as the direct ELISA reading of Herstatin bound to 3T3/HER-2 cells, with the background binding to parental 3T3 cells subtracted.

***Inhibition of growth of DU145 (ErbB receptor family over-expressing) prostate cancer cells***—

On day one, ~40,000 DU145 cells/well in 12-well plates were washed and treated in triplicate with either control vehicle or concentrations from 100 to 300 nM of purified "wt", "R to C," "L/N", or "R to I " Herstatin forms, in media with 0.1% fetal bovine serum. On day 3, the cells were treated a second time and on day 5, viable cells were quantified by a standard MTS cell assay (*e.g.*, as described in Romero-Jhabvala et al., *Oncogene* Vol 22, pp 8178-8186 (2003)). The results (Figures 2 and 3) are plotted as % inhibition relative to vehicle-treated control.

***Shamieh & Clinton***—The Materials and Methods and Results of Shamieh L and Clinton GM, *FEBS Lett*, vol 568, pp163-166 (2004) (attached hereto) are incorporated by reference into this Affidavit, and the methods therein with respect to expression and purification of Herstatin, as well as those pertaining to Herstatin binding, follow the teachings of the present application.

7. *Results.*

**Figure 1** (attached hereto) shows a direct comparison of HER-2-specific binding to HER-2 overexpressing 3T3 cells, among the following allelic variants of Herstatin: the most common form ("wt"); the "L/N" variant (the previously claimed variant 11); and an "R to C" ECDIIIa region variant that is the subject of another pending patent application. <u>All three allelic variants of Herstatin show comparable binding at nM concentrations</u>.

**Figure 2** and **Figure 3** (attached hereto) show a direct comparison of inhibition of the growth of DU145 prostate cancer cells that over-express the ErbB receptor family (including HER-2, EGFR and HER-3 receptors), among the following variants of Herstatin: "wt" (most common sequence); the "L/N","R to C", and "R to I" polymorphic forms of Herstatin. <u>The results show that all three Herstatin allelic forms had strong, comparable activity in inhibiting the growth of this ErbB receptor family-expressing cancer cell line</u>.

**Shamieh L and Clinton** (attached hereto) show binding interactions (using HER-2 overexpressing 3T3 cells and HER-2 transfected Cos-7 cells) of Herstatin and the intron-encoded domain thereof with receptors of the ErbB receptor family including HER-1 (EGFR), HER-2,

HER-3, HER-4, and additionally to IGF-1R. Both "wt" Herstatin (corresponding to present SEQ ID NO:15) and the corresponding intron-encoded portion thereof (corresponding to present SEQ ID NO:14) bind at nM concentrations to HER-2 and to EGFR (and also to ΔEGF-R, HER-4 and IGF-1R). While the initial published results indicate that an "R to I" encoded ECDIIIa allelic variant (Arg –> Ile at amino acid position 31 of the ECDIIIA region, and the subject of another patent application) may eliminate binding of the corresponding intron-encoded portion to HER-2. Our current data (Figure 3) shows, in terms of percent inhibition versus concentration, that the corresponding full-length Herstatin, comprising this variant has dose responsive (100 and 300 nM were tested) bioactivity against ErbB family receptor overexpressing DU145 prostate cancer cells that is comparable to that of the most common "wt" allelic variant. This indicates that in the full length Herstatin protein, the R to I variation does not block receptor interaction required for bioactivity.

8. In conclusion, the data described herein, including that described in Shamieh L and Clinton GM, *FEBS Lett*, vol 568, pp163-166 (2004), which was obtained by following the teachings of the present application, ~~and~~ shows that Herstatin allelic variants, including the most common one (corresponding to SEQ ID NOS:14 and 15), have strong HER-2 binding and activity in inhibiting growth in HER-2 expressing cancer cells that is comparable to the previously claimed polymorphic variant 11. Additionally, the allelic variants have comparable binding and biological activity in EGF expressing cancer cells.

9. I further declare that all statements made herein of my own knowledge are true and that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Gail M. Clinton

State of )
) ss.:
County of )

On this 18th day of January, 2005, before me, a Notary Public in and for the State and County aforesaid, personally appeared Gail M. Clinton, to me known and known to me to be the person of that name, who signed and sealed the foregoing instrument, and she acknowledged the same to be her free act and deed.

Notary Public

Commission expires

FEBS 28495 FEBS Letters 568 (2004) 163–166

# Receptor binding specificities of Herstatin and its intron 8-encoded domain

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**Abstract Retention of intron 8 in alternative HER-2 mRNA generates an inhibitory secreted ligand, Herstatin, with a novel receptor-binding domain (RBD) encoded by the intron. This study examines binding interactions with several receptors and investigates sequence variations in intron 8. The RBD, expressed as a peptide, binds at nM concentrations to HER-2, the EGFR, ΔEGFR, HER-4 and to the IGF-1 receptor, but not to HER-3 nor to the FGF-3 receptor, whereas a rare mutation in the RBD (Arg to Ile) eliminates receptor binding. The full-length Herstatin binds with 3–4-fold higher affinity than its RBD, but with ~10-fold lower affinity to the IGF-IR. Sequence conservation in rhesus monkey but not in rat suggests that intron 8 recently diverged as a receptor-binding module critical for the function of Herstatin.**



*Keywords:* ErbB receptor; HER-1, HER-2, HER-3, HER-4; IGF-I receptor; Herstatin

## 1. Introduction

The ErbB receptor family consists of four receptor tyrosine kinases: EGFR (HER-1, erbB-1), HER-2 (erbB-2), HER-3 (erbB-3) and HER-4 (erbB-4). Aberrant expression of ErbB receptors by mutational activation, receptor overexpression, and tumor production of ligands contributes to the development and maintenance of a variety of human cancers [1,2].

The ErbB receptors are activated by several ligands consisting of an EGF core domain [3]. The exception is the HER-2 receptor, which is recruited as a preferred dimer partner with other ligand binding erbB receptors. While the eleven mammalian EGF-like ligands are all agonists, the ligand Argos, in *Drosophila*, inhibits activation of the EGFR [4,5].

Although the HER-2 receptor does not directly bind EGF-like ligands, a secreted product of an HER-2 alternative transcript, Herstatin, binds with nM affinity to the ectodomain of HER-2. Herstatin consists of a segment of the HER-2 ectodomain followed by 79 novel amino acids, encoded by intron 8, which function as a receptor-binding domain (RBD) [6]. Herstatin blocks homomeric and heteromeric ErbB receptor interactions, inhibits activation of the PI3K/Akt pathway initiated by EGF, TGF-α, and Heregulin, and causes growth arrest suggesting potential as an anti-cancer agent [6–9]. However, no study has yet addressed the receptor specificity of Herstatin. To identify receptor binding targets and to further assess the significance of the novel intron 8-encoded RBD, we investigated binding to several receptors expressed in transfected cells, examined the consequence of a rare mutation in intron 8, and compared the sequence in human, rat and rhesus monkey.

## 2. Materials and methods

### 2.1. Cell lines, transfections, and Western blots

The 3T3/HER-2 cells were previously described [10]. The 3T3/IGF-IR cells were from Dr. Charles Roberts, OHSU, Portland, OR. For transient transfections, 2 μg of empty vector or 2 μg EGFR, HER-2, HER-3, HER-4, ΔEGFR, or FGFR-3-myc expression vectors was added with Lipofectamine (Gibco-BRL) to Cos-7 cells in 6 well plates. The HER-2 and EGFR expression plasmids were previously described [7], ΔEGFR was a gift from Dr. Webster Cavenee (Ludwig Institute, UCSD, La Jolla, CA), the FGFR-3-myc construct was from Dr. William Horton (Shriners Research Hospital, Portland, OR), and the HER-4 expression plasmid was a gift of Dr. Nancy Hynes (Friedrich Miescher-Institute for Biomedical Research, Basel, Switzerland). To analyze receptors by Western blot analysis, proteins were resolved by SDS–PAGE and electro-transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Blots were blocked in 5% milk and incubated with primary antibody overnight at 4 °C. The antibodies included anti-HER-2 [11], anti-EGFR, anti-HER-3, and anti-HER-4, which were all rabbit polyclonal antibodies against the receptor C-terminal domains (Santa Cruz Biotechnology). Antibodies against the β-subunit of IGF-IR were from Dr. Charles Roberts. After washing, the blots were incubated with secondary antibody conjugated to HRP for 30 min (BioRad, Hercules, CA). The membranes were developed with Super-Signal West Dura (Pierce, Rockford, IL) and exposed to X-ray film.

### 2.2. Sequencing of intron 8

Human genomic DNA was obtained from blood samples (supplied by Dr. David Henner, OHSU) from individuals 18 years or more, after giving informed consent, with approval by the Institutional Review Board of OHSU. The samples, assigned random four-digit numbers, could not be traced to patient identity. The polymerase chain reaction (PCR), purification and sequencing were carried out exactly as previously described [6]. Electropherograms were individually reviewed to detect polymorphic alleles. Samples found to contain a polymorphism were sequenced at least twice to confirm the mutation. Rhesus monkey DNA, provided by Dr. Scott Wong (ORPC, Portland, OR), was amplified and sequenced in the same manner. Intron 8 in rat genomic DNA was amplified by PCR using rat specific primers: 5′-CTACCTGTCTACGGAAGTGG-3′ and 5′-TTCCGGGCAGAAATGCCAGG-3′. The cycling parameters were: 94 °C for 30″; 62 °C for 30″; and 72 °C for 60″, for 25 cycles.

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*2.3. Expression and purification of intron 8-encoded peptide (Int8) and Herstatin*

The intron 8 cDNA was cloned into the pET30 bacterial expression vector (Novagen, Madison, WI), expressed in bacteria (BL-21), and purified by nickel affinity chromatography as described [6]. For purification of insect Herstatin, S2 insect cells, stably transfected with 6× His tagged-Herstatin in the pMT/BiP expression plasmid (Invitrogen, Carlsbad, CA), were induced with 100 μM cupric sulfate for ~16 h. Herstatin was purified to ~90% purity by Ni–NTA (Qiagen, Valencia, CA) affinity chromatography as previously described [8].

*2.4. Cell binding studies*

About $2 \times 10^6$ cells in 6-well plates were incubated with purified Herstatin or int8 peptide for 2 h at 4 °C in serum-free media. Cells were washed with phosphate-buffered saline (PBS) and extracted in 50 mM Tris–HCl, pH 7.0, and 1.0% NP-40. Int8 peptide or Herstatin bound to cells was quantified using a sandwich Herstatin ELISA as per the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). The dissociation constant ($K_d$) and maximal binding ($B_{max}$) of Herstatin or the int8 peptide were determined by nonlinear regression analysis of the plot of pmol of bound versus nM of Herstatin or int8 peptide added. Statistical comparisons between different binding curves were performed by extra sums-of-squares *F*-test on nonlinear regression coefficients. All tests were performed ($\alpha = 0.05$) using GraphPad Prism 4 software (GraphPad Software, 1994–2003).

*2.5. Pull-downs with int8 peptide immobilized on protein S agarose*

About 100 μl of a 50% suspension of S-protein agarose (Novagen) was incubated with or without 100 μg of int8 peptide with an S-protein tag, at room temperature for 1 h, and then washed twice with 500 μl PBS. The agarose samples were then incubated at room temperature for 1 h with 200 μg of transfected Cos-7 cell extract and washed twice with 500 μl of PBS with 1% NP40. The proteins were eluted from the resin at 92 °C for 2 min in 40 μl of SDS-sample buffer and analyzed as a Western blot.

## 3. Results

*3.1. Sequence of human, rhesus monkey, and rat intron 8*

Herstatin is generated by retention of HER-2 intron 8, which encodes the unique C-terminal proline-rich domain of 79 amino acids (Fig. 1). Because of its critical function in receptor binding [6], we sequenced genomic HER-2 intron 8 from 214 humans, rhesus monkey, and rat. The HER-2 intron 8 deduced amino acid sequence, originally determined from SKOV3 ovarian cancer cells (AF177761), was found to be the most common in germ line DNA. In addition, we identified a sequence variation in intron 8 (G1112T in AF177761) resulting in an Arg to Ile substitution at residue 31 in Fig. 1. This mutant allele was found in only one of 215 (<0.5%). The deduced amino acid sequence of intron 8 from rhesus monkey was 85% identical to that of humans (Fig. 1) and the nucleotide sequence, up to the stop codon, was 93% identical. However, there was no conservation between rat and human intron 8 (Fig. 1), in contrast to the HER-2 receptor coding sequence, which is highly conserved in rat *neu* [12].

*3.2. Receptor binding of the HER-2 intron 8-encoded peptide*

To identify other potential receptor targets of Herstatin, we examined binding of the intron 8-encoded RBD, expressed as a bacterial peptide (Int8). Protein S agarose, with or without immobilized int8 peptide, was incubated with extracts from Cos-7 cells transiently transfected with several different receptors. Following washing steps, the protein bound to the agarose was analyzed as a Western blot with receptor-specific antibodies. As previously observed [6,7], EGFR and HER-2 from the transfected cell extracts bound specifically to the agarose with

```
Human: 1                                                    I
Human: 1 GTHSLPPRPAAVPVPLRMQPGPAHPVLSFLRPSWDLVSAF40
Rhesus: 1                          C N     L              P L
Rat:    1 GTQPHSKTSLVHPALAstop

Human: 41 YSLPLAPLSPTSVPISPVSVGRGPDPDAHVAVDLSRYEGstop
Rhesus: 41 C     L        M S       DL          N    C   stop
```

Fig. 1. The deduced amino acid sequence encoded by HER-2 (ErbB-2) intron 8. Alignments are with the most common human intron 8 sequence from 214 individuals with non-conserved residues shown.

int8 peptide (Fig. 2A). In contrast, the int8 peptide with the Arg to Ile mutation at residue 31 (see Fig. 1) did not pull-down the HER-2 receptor (Fig. 2B). Fig. 2A also demonstrates that ΔEGFR, a tumor variant of the EGFR missing its N-terminal subdomains I and II [13], specifically associated with int8 peptide. Another member of the erbB family, HER-4, was also pulled-down by int8. However, there was no detectable association of HER-3 with int8 peptide agarose despite abundant expression in the transfected cells (Fig. 2A). We also investigated the possible interaction with the IGF-1 receptor (IGF-IR), which contains regions of ectodomain sequence homology with the EGFR [14]. Interestingly, we observed specific pull-down of the IGF-IR from transfected cell extracts (Fig. 2A). The FGFR-3, a receptor tyrosine kinase with Ig-like motifs and no structural homology with the ErbB family ectodomains, did not bind to the int8 peptide.

To further examine interaction of the int8 peptide with the extracellular domain of receptors at the cell surface, an Herstatin ELISA was used to quantify bound peptide. In agreement with results obtained by the pull-down assay, the int8 peptide bound in a specific and dose-dependent manner to EGFR, HER-2, HER-4, and ΔEGFR, but not to HER-3, FGFR-3, or mock-transfected cells (Fig. 2C). Binding affinities were further characterized by generating saturation-binding curves. Int8 peptide bound to HER-2 transfected Cos-7 cells ($K_d = 50 \pm 6$ nM) and to EGFR transfected Cos-7 cells ($K_d = 78 \pm 10$ nM) with binding affinities, assessed by comparative nonlinear regression analysis, that were not significantly different ($P = 0.40$) (Fig. 3A). Further, int8 peptide bound to the IGF-IR/3T3 cells ($K_d = 70 \pm 21$ nM) and to HER-2/3T3 cells ($K_d = 66 \pm 16$ nM) with similar affinities ($P = 0.96$) (Fig. 3B). In contrast, the mutant int8 peptide with Arg31Ile did not significantly bind to the HER-2 receptor overexpressing cells at any of the peptide concentrations tested (Fig. 3C) even though the Herstatin ELISA detected the wild-type and mutant peptide equally (Fig. 3D). These results suggested that the int8 peptide bound to EGFR, HER-2, and IGF-1R with overlapping binding affinities and that the Arg-Ile mutation inhibited receptor binding without destroying antibody binding epitopes.

*3.3. Receptor binding properties of full-length Herstatin*

The full-length Herstatin bound to 3T3/HER-2 cells with a $K_d = 14.7 \pm 1.8$ nM, which is significantly different from the binding affinity of int8 peptide ($P < 0.0001$) by 3–4-fold. A direct comparison of the binding of Herstatin to 3T3/HER-2 and 3T3/IGF-IR cells revealed that the affinity for the IGF-1R ($K_d \sim 151$ nM) was lower ($P < 0.0001$) by about 10-fold (Fig. 4A). The dissociation constant of Herstatin for EGFR was similar to that of HER-2, and was unaffected by ligand occupation indicated by a $K_d = 16.4 \pm 3.6$ nM versus $16.3 \pm 3.6$ nM (respectively) for Cos-7/EGFR treated or not with 10 nM EGF

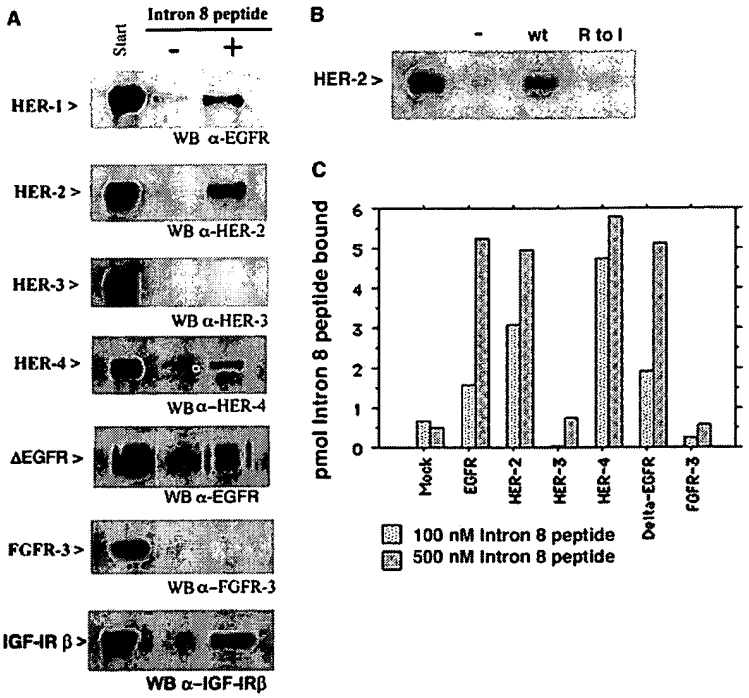


Fig. 2. Binding of intron 8-encoded peptide to different receptors expressed in transfected cells. (A) Extracts from transfected Cos-7 cells were incubated with protein S agarose without or with immobilized wild-type or (B). R31I mutant int8. Associated proteins were analyzed as a Western blot. (C) Transfected Cos-7 cells were incubated with purified int8 for 2 h at 4 °C in serum-free media, cells were washed, extracted, and analyzed by Herstatin ELISA.

(Fig. 4B). Herstatin bound with saturation to endogenous receptors in A431 epidermoid carcinoma cells, which express very high levels of EGFR and low levels of other ErbB receptors (Fig. 4C). At saturation, 6.9 ± 0.4 pmol of Herstatin were bound indicating ~$2 \times 10^6$ binding sites/cell, which matches the number of EGFR per A431 cell at $2 \times 10^6$ [15]. Comparison of nonlinear models indicated that a hyperbolic one affinity-site binding model was the best fit for EGFR-specific binding of Herstatin, in the presence and absence of EGF.

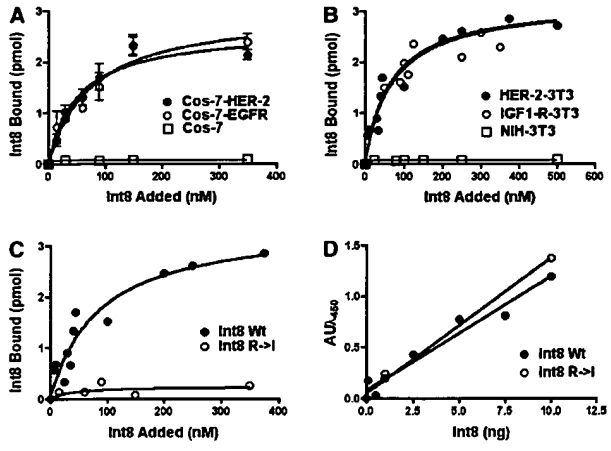


Fig. 3. Saturation binding curves of intron 8 peptide to cells transfected with HER-2, the EGFR, and the IGF-IR. Different amounts of purified int8 were added to the indicated cells and bound peptide was quantified by Herstatin ELISA. Nonlinear regression analysis of binding data was used to determine the dissociation constants ($K_d$) and maximal amount bound. In (A) parental (Cos7) or transiently transfected Cos-7-HER-2 or Cos7-EGFR cells, or in (B) 3T3 cells or stably transfected HER2-3T3 or IGF-IR-3T3 cells were used. In (C) wild-type or R31I mutant Int8 peptides were incubated with HER2-3T3 cells. In (D) indicated amounts of wild-type or R31I peptides were incubated in an Herstatin ELISA.

## 4. Discussion

We present evidence that intron 8 of the HER-2 gene, retained in an alternative HER-2 transcript, encodes a receptor binding domain. We also report that a non-lethal, point mutation of unknown physiological significance, resulting in Arg to Ile in the intron 8-encoded domain, eliminates binding to the HER-2 receptor. Unaltered interaction of this mutant RBD with two monoclonal antibodies in an ELISA suggested that global structure was unaffected and that this Arg residue may be directly involved in receptor binding. While the intron 8 encoded domain is critical for receptor binding, it does not appear to affect receptor activity suggesting a requirement for the N-terminal subdomains I and II of Herstatin for receptor inhibition [6] (Shamieh and Clinton, unpublished observations).

While the intron 8-encoded RBD is critical for the receptor binding activity of Herstatin, it is not conserved between humans and rats despite the high degree of sequence identity between the HER-2 receptor and its rat ortholog, *neu*. There are distinct regions in their ectodomains, however, with very little identity [12]. An additional distinction is that the rat *neu* receptor is activated as an oncogene by a single point mutation in the transmembrane domain, while the human ortholog, HER-2, is oncogenic without aberrations in the coding se-

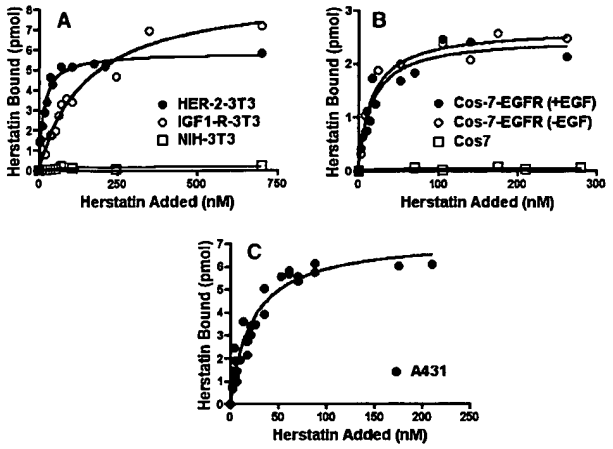


Fig. 4. Saturation binding curves of Herstatin to cells expressing different receptors. Herstatin purified from S2 insect cells was incubated with: (A) 3T3 cells, HER-2-3T3, or IGF-IR-3T3 cells or in (B) with parental or transiently transfected Cos-7-EGFR cells serum starved for 24 h and then treated or not for 2 h on ice with 10 nM EGF, or in (C) A431 epidermoid carcinoma cells.

quence [16]. Furthermore, the activating mutation is not functionally equivalent when introduced into HER-2 [17,18]. These collective observations point to differences in regulation of the human HER-2 receptor versus its rat ortholog, *neu*.

Specific binding of the RBD suggests that the HER-4 receptor will be a target of Herstatin. Since Herstatin binds to and blocks the dimerization of the EGFR and HER-2, we predict that Herstatin will have a similar effect on the structurally similar HER-4. Effects of Herstatin on HER-4 activation and signaling are currently under investigation. Lack of Herstatin binding to the other ErbB family member, the HER-3 receptor, was surprising. HER-3 is unique, however, since it is kinase deficient and requires an active receptor partner to signal. The Herstatin binding site may be disguised when HER-3 is overexpressed without a dimer partner. The binding of Herstatin to the IGF-IR with nM affinity was unforeseen, since ligands do not typically cross-react with receptors from different families. Interestingly, the IGF-IR has regions of ectodomain sequence homology with the EGFR and crosstalk occurs, most notably, with transactivation of the EGFR by IGF-1 [19 and references therein]. Our finding that the binding affinity of Herstatin, but not its RBD, is significantly weaker for IGF-IR than for HER-2 or the EGFR suggests that stabilizing interactions between the N-terminus of Herstatin and the receptor ectodomain are lacking. Since IGF-IR does not have a homologous dimerization loop [14], contacts between the IGF-IR ectodomain and the dimerization arm in subdomain II of Herstatin may be prohibited. The physiological significance of Herstatin binding to the IGF-IR remains to be determined.

In addition to Herstatin, there are several other examples of alternative forms of ErbB receptors that are created by intron read-through [20,21]. Creation of truncated receptors fused to novel C-terminal domains by read-through into introns represents a novel regulatory mechanism important in the diversification of receptor signaling. So far, Herstatin is the only known alternative receptor product that functions as a ligand and is the only mammalian secreted ligand that inhibits the EGF receptor family [18,22,23].

*Acknowledgements:* This work was supported by CA082503 from the NIH. We thank Emily Janega and Dr. Shuhua Guo for providing purified Herstatin from S2 insect cells.

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